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Identification of Putative Velvet Bentgrass Clones Using RAPD Markers

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ABSTRACT

Bentgrasses (*Agrostis* spp.) are extensively used on golf courses in temperate regions for putting greens, tees, and fairways. The three most commonly used bentgrasses, creeping bentgrass (*Agrostis stolonifera* L.), colonial bentgrass (*Agrostis capillaris* L.), and velvet bentgrass (*Agrostis canina* L.), are often difficult to identify on the basis of morphological features. As such, naturalized bentgrass clones collected from old turfs can be difficult to classify. The objective of this study was to determine if random amplified polymorphic DNA (RAPD) markers could identify 319 bentgrass clones according to species, ultimately to identify desirable velvet bentgrass germplasm for breeding projects. Germplasm sources included known velvet, creeping, and colonial bentgrasses, a seed collection of putative velvet bentgrass from the Azores, Portugal, and a clonal collection of putative velvet bentgrass from old Milwaukee golf courses. Five RAPD primers produced 82 polymorphic bands. Analysis of molecular variance (AMOVA) was used to partition variation among groups (14.7%), among populations within groups (16.2%), and among individuals within populations (69.1%). A multidimensional scaling procedure (MDS) differentiated groups according to known species and allocated unknown plants into known clusters. The Milwaukee group was closely associated with creeping bentgrass cultivars while the Azores group associated with velvet bentgrass and colonial bentgrass. Flow cytometry confirmed ploidy levels of known and unknown clones. The clear differentiation between species suggests that RAPD markers are a useful tool for identifying bentgrass species.

Velvet bentgrass was used commercially in the USA during the early 20th century in the seed mixtures known as South German bentgrass (Brilman, 2003). These seed populations usually contained 10 to 15% velvet bentgrass (Piper and Oakley, 1922). The ability of velvet bentgrass to form a fine, dense, bright green turf was attributed to very fine leaf texture and high shoot density (DeFrance et al., 1952). These characteristics resulted in early interest in the use of velvet bentgrass for putting greens (Brilman, 2003).

Reid (1933) demonstrated that velvet bentgrass performs better in shaded environments than creeping bentgrass. Skogley (1975) found that velvet bentgrass quality decreased as fertility rates increased from 15 to 34 g m^{-2} because of summer burn and consequent *Poa annua* L. invasion. DaCosta and Huang (2003b) showed that velvet bentgrass and creeping bentgrass exhibit better turf quality than colonial bentgrass under irrigation regimes which replaced less than 100% of evapotranspirational water loss. In a growth chamber study, velvet bentgrass was more drought tolerant than creeping bentgrass on the basis of turf quality, leaf relative water content, and root number and viability during water stress (DaCosta and Huang, 2003a). Tolerance of velvet bentgrass to lower levels of light, fertility, and water, makes it a superior choice for breeders looking to develop a high quality turf able to tolerate reduced inputs. Traditionally velvet bentgrass has been perceived as a high-maintenance turfgrass species that requires a temperate oceanic climate and low soil pH to survive (Reid, 1932; Christians, 1998; Turgeon, 1999). Velvet bentgrass turf use largely ceased after the mid-20th century because of seed production problems, thatch management issues, and the introduction of seeded creeping bentgrasses (Brilman and Meyer, 2000). Current seed production and certification procedures ensure that turfgrass seed is relatively pure and weed free. Advances in thatch management on putting greens using aeration and topdressing may nullify previously observed thatch problems. Collections of velvet bentgrass from natural areas or old golf courses outside of temperate oceanic climates could yield clones adapted to local environments, including higher soil pH and more variable temperatures.

Identification of *Agrostis* spp. on the basis of the morphology of vegetative and reproductive structures is tedious and difficult. Molecular marker technology has the potential to assist species identification. A study of grain amaranths (*Amaranthus* spp.) using RAPD markers efficiently classified accessions by species and identified grain amaranths misclassified by morphology (Transue et al., 1994). Scheef et al. (2003) created sequence characterized amplified region (SCAR) markers which accurately differentiated between creeping bentgrass, colonial bentgrass, and artificial interspecific hybrids of creeping bentgrass and colonial bentgrass. Laser flow cytometry reliably classified fine fescue species (*Festuca* spp.) on the basis of chromosome number (Huff and Palazzo, 1998) and successfully determined the ploidy level of bentgrasses (Bonos et al., 2002). Since velvet bentgrass is diploid, while creeping and colonial bentgrass are tetraploid, DNA content or chromosome number can effectively provide the means to distinguish diploid from tetraploid individuals.

Genetic diversity within a species may also be characterized by RAPD markers. Casler et al. (2003) employed RAPD markers to distinguish among creeping bentgrass clones collected from Wisconsin golf courses. Gunter et al. (1996) utilized RAPD markers to evaluate genetic diversity among populations of switchgrass (*Panicum virgatum* L.). Cultivar identification by RAPD markers has been successful in creeping bentgrass (Golembiew-

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ski et al., 1997) and buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.] (Wu and Lin, 1994). Knowledge of germplasm diversity and genetic variability of a species enhances the effectiveness of breeding and conservation of germplasm.

The objective of this research was to evaluate the genetic diversity of bentgrass collections and determine if RAPD markers could be utilized to classify bentgrass germplasm collected from old golf courses and natural areas according to bentgrass species.

MATERIALS AND METHODS

Germplasm

Bentgrasses used in this study represented seven groups: Milwaukee, Seed Research of Oregon (SRO), Azores, Plant Introductions (PI), Velvet, Creeping, and Colonial (Table 1). The Milwaukee group consisted of putative velvet bentgrass clones collected from the putting greens of golf courses in urban areas of Milwaukee, WI, in August 2000. The 12 golf courses were chosen as collection sites because they were established in the early part of the 20th century and they were originally seeded with South German bentgrass. Clones were selected on the basis of fine leaf texture, high shoot density, and patch uniformity. A single stolon was selected from a plug collected from each clone and was grown under greenhouse conditions until sampled for analysis.

The SRO group consisted of 19 seeded velvet bentgrass breeding lines from Seed Research of Oregon. Each breeding line derived from an old golf course in the northeastern USA. Most of these original collections were made in the early 1990s.

The Azores group consisted of putative velvet bentgrass clones derived by sampling random panicles from natural meadows on the islands of São Miguel, Terceira, and Pico in the Azores, Portugal. Collections Az-SM1 and Az-SM2 were made from two meadows at the top of the crater overlooking Lago Do Fogo on São Miguel. The altitude was 950 m and the plants were conspecific with other plants of the genera *Trifolium*, *Lotus*, *Holcus*, *Digitaria*, and *Sporobolus*. Collection Az-P was made in a rocky meadow along the hiking path to the top of Pico volcano on the island of Pico. The altitude was 1200 m and the plants were conspecific with *Holcus mollis* L., *Holcus* spp., and *Festuca jubata* Lowe. Collection Az-T was made in a wet pasture 500 m east of highway V.R. midway between junctions with highways 5-2a and 3-2a on the island

Table 1. Bentgrasses group and population collection and location information.

Group	Population	Collection site	Location	No. of clones
Milwaukee	BYCC	Brynwood CC	Milwaukee, WI	12
	WBCC	West Bend CC	West Bend, WI	5
	SCC	Squires CC	Port Washington, WI	8
	BDP	Brown Deer Park	Milwaukee, WI	1
	OzCC	Ozaukee CC	Mequon, WI	30
	TCC	Tripoli CC	Milwaukee, WI	20
	WCC	Westmoor CC	Brookfield, WI	13
	BMGCC	Blue Mound G&CC	Wauwatosa, WI	32
	NHCC	North Hills CC	Menomonee Falls, WI	9
	CCC	Chenequa CC	Hartland, WI	10
	HGC	Hartford GC	Hartford, WI	21
	LLBCC	Lac La Belle CC	Oconomowoc, WI	1
SRO†	P25-32	Green Harbor CC	Massachusetts	5
	P23-32	Portsmouth Pasture	New Hampshire	5
	P24-32	Green Harbor CC	Massachusetts	5
	P23-31	Area VI Univ. R.I.	Rhode Island	5
	P85-10	Martindale GC	Maine	5
	P84-10	S. Portland Municipal GC	Maine	5
	VAL	Valhala GC	Maine	5
	P86-7	Valhala GC	Maine	5
	P86-10	Martindale GC	Maine	5
	P86-3	Poland Spring GC	Maine	5
	B35-20	Upstate New York	New York	5
	P85-6	Valhala GC	Maine	5
	P85-5	Poland Spring GC	Maine	5
	P85-2	Poland Spring GC	Maine	5
	P79-9	Valhala GC	Maine	5
	P86-5	Poland Spring GC	Maine	5
	P81-9	Martindale GC	Maine	5
	P80-9	Valhala GC	Maine	5
	P85-9	S. Portland Municipal GC	Maine	5
Azores	Az-SM1	São Miguel	Azores, Portugal	2
	Az-SM2	São Miguel	Azores, Portugal	1
	Az-T	Terceira	Azores, Portugal	6
	Az-P	Pico	Azores, Portugal	5
Velvet	Vesper	Pickseed	New Brunswick, NJ	4
	Bavaria	Turf Merchants	Northern Europe	4
Creeping	Providence	Seed Research of Oregon	North America	4
	Pennlinks	Pennsylvania State Univ.	Shaker Heights, OH	4
	Penncross	Pennsylvania State Univ.	North America	4
Colonial	Alister	Turf-Seed	North America	4
	SR7150	Seed Research of Oregon	Beltsville, MD	4
	EW	Seed Research of Oregon	North America	8
PI‡	PI 189141	Novobent (cv.)	Netherlands	3
	PI 194697	Barenbrug	Netherlands	3
	PI 290707	University of Reading	UK, England	3
	PI 578526	Kingstown (cv.)	Rhode Island, USA	3

† SRO: Seed Research of Oregon breeding lines.
‡ PI: *Agrostis canina* Plant Introductions from the USDA National Plant Germplasm System.

of Terceira. The altitude was 200 m and the collection was made from an apparent monoculture of *Agrostis*.

The PI group consisted of plant introductions identified as *Agrostis canina* from the USDA National Plant Germplasm System (NPGS). Six PIs were obtained, but only four were used because two displayed morphological features inconsistent with the stoloniferous growth habit of velvet bentgrass (Christians, 1998; Turgeon, 1999).

The Velvet group consisted of seeded velvet bentgrass cultivars Vesper and Bavaria. The Creeping group consisted of seeded creeping bentgrass cultivars Providence, Pennlinks, and Penncross. The Colonial group consisted of the seeded colonial bentgrass cultivars Alister and SR7150 and eight colonial bentgrass clones from the northeastern United States (EW).

Individual plants were established from a single seed or stolon in 12 cm diameter pots. The potting medium was Metro-Mix 366-P (Scott-Sierra Horticultural Products Company, Marysville, OH). Plants were grown under greenhouse conditions from August 2001 to January 2004 with a minimum of 400 μmol m^{-2} s^{-1} of photosynthetically active radiation and a 16-hour photoperiod augmented with high-pressure sodium lamps (model HPS1000, Sunlight Supply Inc., Vancouver, WA). Plants were irrigated with tap water (pH 7.5) as needed to prevent wilting, fertilized weekly with a soluble fertilizer (Technigro 20-9-20, Sun Gro, Bellevue, WA) at 0.348 g L^{-1} and trimmed and thinned as necessary to maintain plant vigor.

Genomic DNA Extraction and Analysis

Fresh leaf tissue (0.1–0.2 g) was collected from individual greenhouse-grown plants and ground in 600 μL of potassium ethyl xanthogenate (PEX) DNA extraction buffer in 2.0-mL screw-cap microcentrifuge tubes using ceramic beads and a FastPrep FP120 (BIO 101 Inc.; Carlsbad, CA) set at speed 5.0 for three 20-s runs. All remaining DNA extraction steps were conducted in 1.5-mL microcentrifuge tubes and followed the procedure of Johns et al. (1997) and Scheef et al. (2003).

Reactions for RAPD analysis were conducted in 10-μL volumes in 0.2-mL thin-walled 8-tube strips in a GeneMate Genius thermocycler (ISC Bioexpress, Kaysville, UT). Polymerase chain reaction cycling parameters followed the procedure of Johns et al. (1997). A set of 32 plants representing all groups was prescreened for polymorphic bands by seven RAPD primers (Operon Technologies, Alameda, CA). Reaction products were electrophoresed at room temperature on 1.5% (w/v) agarose gels for 2 h at 180 V, stained with ethidium bromide, illuminated by UV light, and a digital image was collected with an Epson PhotoPC 3100z (Epson America, Inc. Long Beach, CA). Out of seven RAPD primers, five (A-13, G-19, O-6, P-8, and Y-5) were used for the study because they produced 82 repeatable polymorphic bands (Table 2). Bold and clear bands (Skroch and Nienhuis, 1995) were manually scored for presence or absence by the software program GeneTools (Syngne Inc. Frederick, MD) to track band scoring.

Analysis of molecular variance (AMOVA) was used to partition variation among groups, among populations within groups and among individuals within populations (Excoffier et al., 1992). The among-population portion of the AMOVA was partitioned into six orthogonal single-degree-of-freedom contrasts by partitioning the sum of squares for groups. A multidimensional scaling procedure (MDS) was used to fit the 319 $\times$ 319 genetic distance matrix into two-dimensional coordinates (SAS PROC MDS; SAS Institute, 1990).

Table 2. Random amplified polymorphic DNA (RAPD) primers used to evaluate 319 bentgrass clones.

Name	Sequence (5′→3′)	Number of polymorphic bands	Size range
			bp
A-13	CAGCACCCAC	21	400–1500
G-19	GTCAGGGCAA	14	430–1100
O-6	CCACGGGAGG	16	350–1700
P-8	ACATCGCCCA	16	450–1300
Y-5	GGCTGCGACA	15	580–1370

Flow Cytometry

Fresh leaf tissue was collected from a sample of 14 bentgrass clones representing both known and unknown *Agrostis* spp. The youngest fully expand soybean [*Glycine max* (L.) Merr.] leaf from the cultivar Belle was used as the standard because Arumuganathan and Earle (1991a) reported it has a DNA content of 2.31 pg DNA/2C, which is less than the DNA content of velvet bentgrass (3.42 pg DNA/2C), creeping bentgrass (5.27 pg DNA/2C), and colonial bentgrass (5.87 pg DNA/2C) (Bonos et al., 2002). The nuclear DNA content of *Agrostis* spp. was determined by the protocol of Arumuganathan and Earle (1991b) with a few modifications. Approximately 55 mg of bentgrass leaf tissue along with 5 mg of soybean leaf tissue was chopped in plastic Petri dishes on ice in 1 mL of solution A. The homogenate was filtered through a 30-μm nylon mesh screen into a 1.5-mL microcentrifuge tube. The tubes were centrifuged for 1 min at 13 200 rpm, following which the supernatant was poured off. The pellet was resuspended in 1 mL of solution A by vortexing. The tubes were centrifuged a second time for 1 min at 13 200 rpm, following which the supernatant was poured off. The pellet was then resuspended in 400 μL of solution B that did not contain chicken red blood cells. Tubes were incubated in a 37°C water bath for 15 min. Following incubation, tubes were stored in a 4°C refrigerator or on ice until analyzed. Analysis was done at the Flow Cytometry Facility of University of Wisconsin Comprehensive Cancer Center using a FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA) with a 488-nm laser. For each sample 10 000 nuclei were collected and analyzed. ModFitLT version 3.0 was used to create a histogram of the data. Plant nuclear 2C DNA content was determined by multiplying the ratio of the *Agrostis* peak to the standard peak by 2.31 pg (known nuclear DNA content of soybean). Replicated measurements of separate nuclei extractions were used to confirm the reproducibility of results.

RESULTS AND DISCUSSION

An analysis of molecular variance among the seven groups, as well as between and within the populations of these groups, showed the majority of genetic variance came from within populations. Only 14.7 and 16.2% of the variance was attributed to differences among groups and differences between populations of these groups, respectively (Table 3). This is consistent with other studies of outcrossing species, which showed the majority of genetic variance was found among individuals within the populations (Diaby and Casler, 2003; Huff et al., 1993; Huff, 1997; Ubi et al., 2003).

Partitioning of the variance among groups revealed that the majority of variance came from differences among species (Table 3). Because markers were chosen on the basis of groups, this result was expected. Differences between the creeping bentgrass and colonial bentgrass groups were slightly significant ($P = 0.0978$) and

Table 3. Analysis of molecular variance (AMOVA) for 82 random amplified polymorphic DNA (RAPD) bands and 319 bentgrass clones.

Source of variation	df	Sum of squares	*P* value	Variance component	Percentage of variation	Percentage (within level)
Groups	**6**	**1005.94**	**<0.0001**			**100.0**
Creeping cv. & Colonial cv. vs. all others	**1**	**93.87**	**0.0821**	**1.09**	**1.1**	**7.2**
Creeping vs. colonial	**1**	**71.92**	**0.0978**	**3.88**	**3.8**	**25.8**
Velvet cv. & PIs vs. Milwaukee, SRO & Azores	**1**	**133.195**	**0.0049**	**2.80**	**2.7**	**18.6**
Velvet cv. vs. Velvet PIs	**1**	**22.592**	**0.4839**	**0.13**	**0.1**	**0.9**
SRO vs. Milwaukee & Azores	**1**	**599.573**	**<0.0001**	**4.59**	**4.5**	**30.5**
Milwaukee vs. Azores	**1**	**84.798**	**<0.0001**	**2.57**	**2.5**	**17.1**
Populations within groups	**40**	**984.62**				**100.0**
Milwaukee golf courses	**11**	**286.07**	**<0.0001**	**1.15**	**1.1**	**7.0**
Azores collections	**3**	**53.57**	**0.0235**	**1.64**	**1.6**	**9.9**
Plant introductions	**3**	**60.33**	**<0.0001**	**3.70**	**3.6**	**22.3**
SRO families	**18**	**492.06**	**<0.0001**	**3.63**	**3.5**	**21.9**
Velvet cultivars	**1**	**20.38**	**0.0362**	**2.46**	**2.4**	**14.8**
Creeping cultivars	**2**	**32.83**	**0.0020**	**1.92**	**1.9**	**11.6**
Colonial cultivars	**2**	**39.38**	**<0.0001**	**2.07**	**2.0**	**12.5**
Clones within populations	**272**	**2840.06**				**100.0**
Milwaukee golf courses	**150**	**1677.40**	**<0.0001**	**11.18**	**10.9**	**15.8**
Azores collections	**10**	**127.93**	**0.0235**	**12.79**	**12.4**	**18.1**
Plant Introductions	**8**	**72.00**	**<0.0001**	**9.00**	**8.8**	**12.7**
SRO families	**76**	**699.60**	**<0.0001**	**9.21**	**9.0**	**13.0**
Velvet cultivars	**6**	**63.25**	**0.0362**	**10.54**	**10.3**	**14.9**
Creeping cultivars	**9**	**78.50**	**0.0020**	**8.72**	**8.5**	**12.3**
Colonial cultivars	**13**	**121.38**	**<0.0001**	**9.34**	**9.1**	**13.2**
Total	**318**	**4830.61**		**102.77**	**100.0**	

contributed 25.8% to the variation among groups. The small sample size of both groups can account for the lack of a more significant difference.

Differences between the known velvet bentgrass groups (cultivars and PIs) and the unknown collections (Milwaukee, Azores, and SRO) were significantly different ($P = 0.0049$) and contributed 18.6% to variation among groups. Because the majority of unknown clones have banding patterns similar to known creeping bentgrass clones, the majority of these clones appear to be genetically different from known velvet bentgrass. Only 0.9% of the variation among groups was attributed to differences between the velvet bentgrass cultivar group and the PI group. The small amount of variation contributed by the difference between velvet bentgrass cultivars and PI group was nonsignificant ($P = 0.4839$) and the plant introductions had RAPD marker profiles consistent with velvet bentgrass. The difference between the SRO group and the Milwaukee and Azores collection was highly significant ($P < 0.0001$) and accounted for 30.5% of the variation among groups. The SRO group is composed of velvet bentgrass breeding lines, which makes it likely that the Milwaukee and Azores collection are composed mostly of other bentgrass taxa. Differences between the Milwaukee and Azores groups contributed 17.0% to variation among groups, suggesting that these two groups also contain different species.

Partitioning of the variance among populations within groups revealed that the Milwaukee and Azores populations contributed the least variation while PI and SRO populations contributed the most variation (Table 3). This low level of variation was attributed to the lack of population clustering within the Milwaukee and Azores groups. Clones within the Milwaukee and Azores populations were more variable than the populations themselves, indicating relatively little divergence among geographic collection sites within these two regions.

The PI and velvet populations contributed a greater percentage of variation among populations within groups (14.8 and 22.3% respectively), than did the creeping and colonial populations (11.6 and 12.5% respectively). As a result of this observation, analyses of molecular variance were also conducted with known cultivars to determine if the variation among and within populations for the three bentgrass species was similar (Table 4). The variation among populations was 25.5, 18.1, and 29.2% for

Table 4. Analyses of molecular variance (AMOVA) for 82 randomly amplified polymorphic DNA (RAPD) bands of known cultivars within three bentgrass species.

		Species			
Source of Variation	df	Sum of squares	*P* value	Variance components	Percentage of variation
			Velvet bentgrass		
Among populations	**5**	**103.30**	**0.0362**	**3.31**	**25.5**
Within populations	**14**	**135.25**	**0.0362**	**9.66**	**74.5**
Total	**19**	**238.55**		**12.97**	
			Creeping bentgrass		
Among populations	**2**	**32.83**	**0.0196**	**1.92**	**18.1**
Within populations	**9**	**78.50**	**0.0196**	**8.72**	**81.9**
Total	**11**	**111.33**		**10.64**	
			Colonial bentgrass		
Among populations	**1**	**22.62**	**<0.0001**	**3.52**	**29.2**
Within populations	**6**	**51.25**	**<0.0001**	**8.54**	**70.8**
Total	**7**	**73.87**		**12.06**	

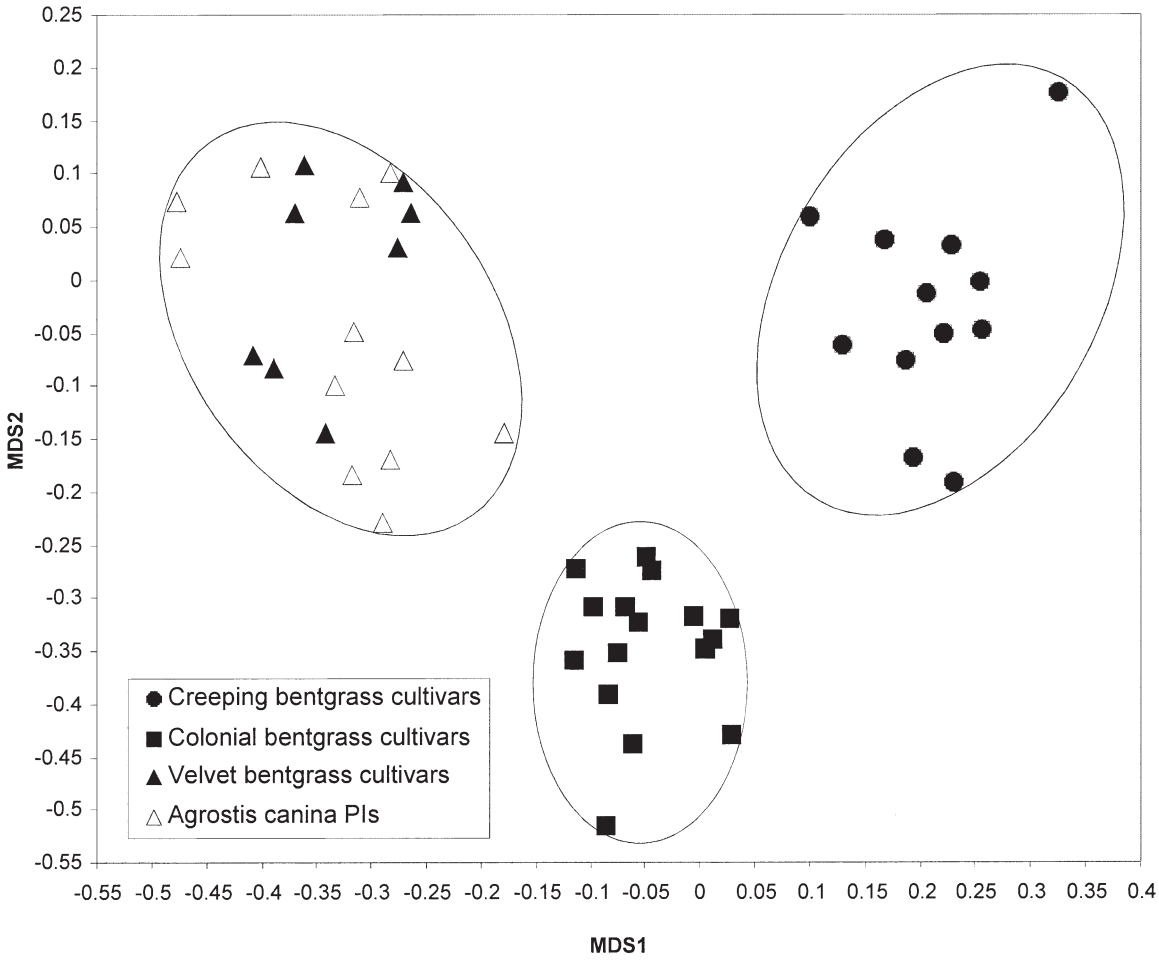

Fig. 1. Scatterplot of the first two multidimensional scales (MDS) for 48 clones from known velvet, creeping, and colonial bentgrass cultivars evaluated for 82 random amplified polymorphic DNA (RAPD) markers.

velvet bentgrass, creeping bentgrass, and colonial bentgrass, respectively. This agrees with studies of perennial ryegrass, *Lolium perenne* L. (Huff, 1997) and buffalograss (Huff et al., 1993), showing the majority of variation in outcrossing species occurs within populations due to heterogeneity between individuals, not differences among populations. The similar levels of variation among populations for the three bentgrass species indicated that differences in ploidy and/or breeding history among these species have not significantly altered their population genetic structure.

A scatterplot of the first two multidimensional scales separated the 48 known clones into three main clusters (Fig. 1). The three clusters corresponded to known creeping bentgrass cultivars, colonial bentgrass cultivars, and velvet bentgrass cultivars plus *Agrostis canina* PIs. The creeping bentgrass cultivars tended to cluster at above-average values of MDS1. The known velvet bentgrass cultivars and four PIs clustered at below-average values of MDS1 and at values of MDS2 greater than −0.25. The colonial bentgrass cultivars and breeding lines tended to cluster at below-average values of MDS1 and at MDS2 values below −0.25. Differentiation among the three groups of known cultivars was complete and unequivocal. Flow cytometry confirmed the ploidy level of all clones sampled from within these three clusters. Clones sampled from a velvet cultivar and PI had 3.16 and 3.19 pg nuclear 2C DNA respectively, while clones sampled from creeping and colonial bentgrass cultivars had 6.35 and 6.86 pg nuclear 2C DNA. Although this difference between the diploid and tetraploid bentgrass species is more than noted by previous studies (Bonos et al., 2002), the ratio is nearly equal to the expected value of 2.

All but one of the putative velvet bentgrass clones from Milwaukee grouped within the region of the cluster of creeping bentgrass cultivars (Fig. 2). Because of the clear distinction between the creeping and velvet bentgrass cultivar clusters, it is most likely that these putative velvet bentgrasses were actually fine-textured creeping bentgrasses. Three plants in the Milwaukee collection, two from Blue Mound Golf & Country Club (BMGCC) and one from Tripoli Country Club (TCC), were previously classified as velvet bentgrasses based on morphological features at anthesis (L. Brilman, 2003, personal communication). Both clones from BMGCC had banding patterns similar to creeping bentgrass while the clone from TCC had a banding pattern similar to the known velvet bentgrasses. Flow cytometry of these three bentgrasses agreed with the cluster analysis. The clone from TCC had 3.16 pg nuclear 2C DNA while the two

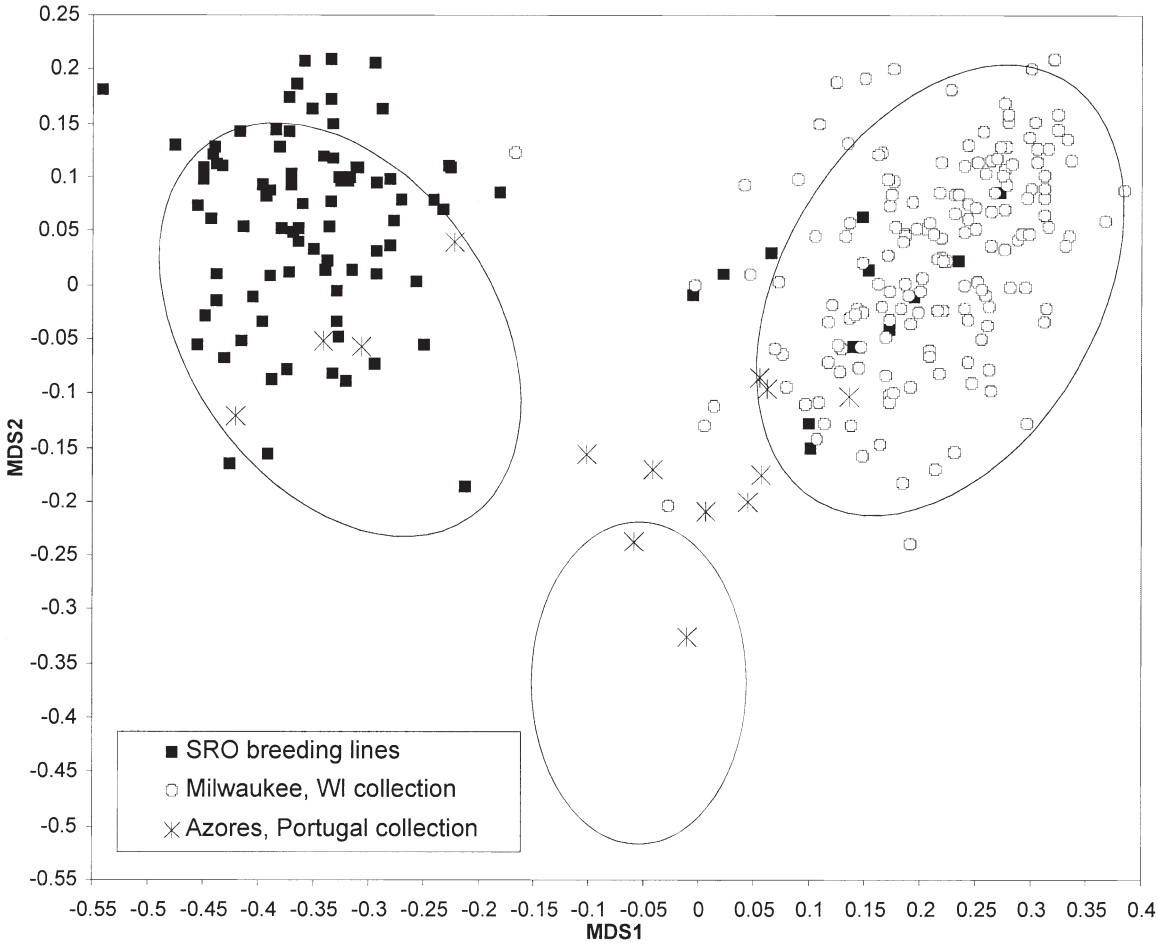

Fig. 2. Scatterplot of the first two multidimensional scales (MDS) for 271 unknown clones evaluated for 82 random amplified polymorphic DNA (RAPD) markers. Ellipses represent the areas where known species clustered in Fig. 1.

clones from BMGCC had 5.96 and 6.14 pg nuclear 2C DNA. Another clone from BMGCC was also analyzed and had 5.98 pg nuclear 2C DNA. Although only one clone from the 162 clones in the Milwaukee group was identified as a velvet bentgrass, there is a possibility that velvet bentgrasses were collected, but were not sampled when a single stolon was selected from each 1-cm diameter sod plug for DNA extraction, particularly if velvet bentgrass stolons are less aggressive or competitive than creeping bentgrass stolons.

The collection of putative velvet bentgrass clones from the Azores segregated into two clusters (Fig. 2). One cluster was closely associated with the known velvet bentgrasses, while the other cluster formed a bridge between the known colonial bentgrasses and the known creeping bentgrasses. In some cases, individual plants from Terceira and São Miguel grouped with either colonial or creeping bentgrasses. Because these collections were developed by sampling random panicles in natural meadows, it is likely that these meadows are mixtures of velvet bentgrass, creeping bentgrass, and colonial bentgrass, resulting in seed collections of multiple species. These meadows may also include some hybrids between creeping and colonial bentgrass, as indicated by the intermediate RAPD marker profiles of several of these clones. Examination of individual plants revealed a number of plants from the Terceira collection had developed rhizomes while growing in the greenhouse, a characteristic of colonial bentgrass (Christians, 1998). Flow cytometry on two plants from Terceira showed that the clone associating with velvet bentgrass and the clone associating with colonial bentgrass had respectively, 3.12 and 6.21 pg nuclear 2C DNA, confirming that these panicle collections were from meadows where diploid and tetraploid bentgrasses were sympatric.

Sixteen of the 19 SRO breeding lines clustered with the velvet bentgrass cultivars (Fig. 2). Flow cytometry demonstrated that a clone from P85-6, which clustered with velvet bentgrass, had 3.05 pg nuclear 2C DNA. All clones within populations P81-9 and P80-9 and two clones within population B35-20 clustered closely with the creeping bentgrass cultivars. The clones that segregated with creeping bentgrass had a vigorous and prolific stoloniferous growth habit that resembled clones from the Milwaukee collection more closely than clones from the other velvet bentgrass breeding lines. Populations P81-9 and P80-9 are most likely creeping bentgrasses that were classified as velvet bentgrass because of their extremely fine leaf texture, high shoot density, and short internode length. Population B35-20 could contain creeping bentgrass plants or represent a hybrid

between the two species. Flow cytometry indicated that individual clones from P81-9, P80-9, and B35-20 had 5.98, 6.03, and 6.10 pg nuclear 2C DNA, respectively, confirming that these clones are tetraploid and likely belong to creeping bentgrass or represent tetraploid hybrids between velvet and creeping bentgrass.

Belanger et al. (2003a) demonstrated that fertile hybrids between creeping bentgrass and velvet bentgrasses occurred when flowering time was manipulated, but the frequency of occurrence was less than or equal to the frequency of self pollination (0.2–0.5%). In a field experiment, interspecific hybrids of velvet bentgrass and creeping bentgrass were not recovered but hybrids of creeping bentgrass and colonial bentgrass were recovered at a frequency of 0.044% (Belanger et al., 2003*b*). They observed that velvet bentgrass finished flowering before creeping bentgrass began to shed pollen, making the possibility of hybrids unlikely in the wild.

The current genomic designation for *Agrostis* lists velvet bentgrass ($2n = 2x = 14$) as C_1C_1, creeping bentgrass ($2n = 4x = 28$) as C_2C_2SS, and colonial bentgrass ($2n = 4x = 28$) as $C_1C_1C_2C_2$ (Warnke, 2003). Vergara and Bughrara (2003) demonstrated that these three species belong to distinct genetic groups on the basis of amplified fragment length polymorphism (AFLP) markers, which showed that creeping and colonial bentgrass are more similar to each other than each is to velvet bentgrass. Our results with RAPD markers further support these genomic designations and taxonomic relationships. Gene flow among bentgrass species has occurred in the past and likely continues to occur in natural areas such as the *Agrostis*-containing meadows of the Azores.

SUMMARY AND CONCLUSIONS

Bentgrass breeders rely heavily on germplasm exploration, utilizing collections from old turfs and natural areas for germplasm enhancement and cultivar development. Natural variation present on old golf courses has been extremely useful as the foundation of several creeping bentgrass breeding programs. However, positive identification of collections is an important first step toward the utilization of germplasm in a breeding program. Incorrect classification can result in meiotic abnormalities, sterility, and inbreeding, slowing breeding progress. Morphological traits are not entirely reliable for timely classification of unknown *Agrostis* germplasm to be utilized in a breeding program, which often requires manipulation of light, temperature, and photoperiod to induce flowering.

The RAPD markers clearly delineated plants of known velvet, creeping, and colonial bentgrass populations. Collections of putative velvet bentgrasses yielded variable results, suggesting the collections were a mixture of species or interspecific hybrids, possibly involving colonial bentgrass, which shares genomes with both *A. canina* and *A. stolonifera*. The majority of variance occurred within populations rather than among populations, as previously demonstrated for populations of numerous outcrossing perennial grasses. The clear separation observed among bentgrass species provides strong support for the use of RAPD markers for species identification within the *Agrostis* genus.

The limited success of collecting velvet bentgrass from Milwaukee golf courses planted with South German bentgrass suggests a number of possibilities. Collecting bentgrass from areas where South German bentgrass was planted does not guarantee the presence of velvet bentgrass populations 75 to 100 yr later. Furthermore, collecting bentgrasses on the basis of morphology does not certify that velvet bentgrass will be selected. By collecting plugs that contain 100 or more tillers and only selecting one tiller for analysis, collected velvet bentgrass clones may have been discarded. Multiple-tiller samples of suspected velvet bentgrass patches should be used to capture this potentially valuable germplasm.

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